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PRINCIPAL INVESTIGATOR: Charles Fehr

CONTRACTING ORGANIZATION: The University of Arizona
Tucson, Arizona 85719

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Tucson, Arizona 85719

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Using a partial-length bovine cDNA probe, a human cDNA encoding the 115kD subunit of the vacuolar type ATPase proton pump has been cloned and sequenced from a human pancreas cDNA library. The sequence contains an open reading frame of 2496 nucleotides, predicting a protein of 831 amino acids with a native molecular weight of 110-120 kD. The predicted primary structure shows 97% and 95% identity to the bovine a and rat amino acid sequences, respectively. A 45% identity is present in comparison to a recently reported osteoclast-specific human 115kD subunit sequence, although hydropathy plots of all reported 115kD sequences are nearly identical. PCR analysis of 28 human tissue cDNA libraries indicates that two alternative isoforms of this subunit exist. The alternative forms were detected only in neural tissues, consistent with previously demonstrated alternative splicing of this protein in human brain. Generation of antibodies targeting all predicted hydrophillic domains of the 115kD subunit is in progress.

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PI - Signature 9/17/97 Date

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INTRODUCTION

Vacuolar type ATPases are ubiquitous, heteromultimeric proteins which translocate protons across cellular membranes and are largely responsible for the acidification of lysosomes, endosomes, and other cellular vesicles. It has been demonstated that some cancerous and proliferative cell types, including a number of breast cancer cell lines, functionally express V-ATPases at the plasma membrane (Gillies *et al.*1992), enabling these cells to survive and grow in unusually acidic conditions associated with some tumors and poorly vascularized tissues. However, attempts to detect VATPase at the plasma membrane by immunocytochemistry and biotinylation have been unsuccessful. In order to investigate plasma membrane expression of V-ATPases in these systems, antibodies against extracellular epitopes are needed. The 115kD subunit is an integral membrane component of the predominantly cytosolic pump complex, and the only subunit with significant extracellular epitopes. The function of this protein is unknown, but antibodies directed against it will be useful in future studies of the subunit and V-ATPase expression. Identification of potentially useful epitopes and synthesis of fusion proteins for antibody generation is dependant upon identification of the human sequence encoding the 115kD subunit.

Previously, it was demonstrated that two isoforms of the bovine 115kD subunit exist as a result of alternative splicing (Peng *et al.*1994). PCR analysis of various bovine tissue cDNA libraries indicated that brain tissue contained an alternatively spliced mRNA with an 18 base insertion. The resulting 6 amino acid sequence comprises a putative, protease-sensitive PEST site. Only the brain library displayed this larger isoform.

Here we have used a partial length bovine 115kD cDNA to isolate clones from a human pancreas cDNA library. Thirteen positive clones were isolated, and five were subsequently used to generate the human sequence. Using this sequence information, PCR primers were generated flanking the site of alternative splicing seen in bovine brain. PCR analysis of 28 human tissue cDNA libraries demonstrated that alternative PCR products were generated from some brain tissue libraries, but not from other tissue type libraries, indicating a pattern of isoform expression similar to that demonstrated in bovine tissues.

BODY

Materials and Methods

Library Screening

A human pancreas cDNA library (PA12948#5 Clontech Labs, Palo Alto,CA) in the Lambda ZapII vector (Stratagene) was screened using a double filter lift procedure. Plaque transfer from NZYDT agar to nitrocellulose filter was performed at room temperature for 5 minutes per filter. After 2 hours at 80C, the filters were prehybridized in 2X SSC, 35% formamide, 0.1% SDS, and 1mg/ml salmon sperm DNA for 8 hours at 42C. Hybridization was performed using a ^{32}P-dATP random-labelled 2.0kb fragment of the bovine 115kD cDNA (a generous gift of Dennis Stone, University of Texas SW Medical Center, Dallas, TX) in 2X SSC, 35% formamide, 0.1% SDS, and 1mg/ml salmon sperm DNA for 24 hours at 42C. Clones which were positive on both filters were excised and two additional rounds of screening were used to isolate and purify them. The Ex-assist helper phage was used to rescue inserts from 13 positive clones into pBluescript.

Sequencing

All 13 clones were initially sequenced using T_3 and T_7- primed dideoxy sequencing, generating sequences corresponding to the 200-300 base pairs at both terminals of each clone. This information was used to map the clones' position relative to the known bovine sequence. Five overlapping clones were subsequently used for further sequencing. Diagnostic restriction digestions were performed to determine subcloning parameters. All subcloning was done using pBluescript and subclones were sequenced using T_3 and T_7 primers. Sequence-specific oligonucleotide primers were synthesized for sequencing regions not sequenced by direct use of T_3 and T_7 primers.

PCR of Human Libraries

PCR primers were designed to flank the site corresponding to that of bovine alternative splicing. Sense primer, 5'ggaactctcaactttggtgggat3', corresponding to nucleotides 2174-2197 and antisense primer- 5'aagctcttcacgctcaggccgat3', corresponding to nucleotides 2457-2435 both contained 5' EcoR-1 cut sites for subsequent ligation into pBluescript and sequencing of PCR products. Hot start PCR reactions were started at 94C for 5 minutes, with 28 subsequent cycles run under the following conditions: denaturation 94C for 1 minute, annealing 62C for 1 minute, and elongation 72C for 1 minute. PCR products were electrophoresed in 5% polyacrylamide, stained with ethidium bromide, excised, ligated into pBluescript via EcoR-1 restriction, and sequenced using T_3 and T_7 priming.

Results

Screening of a human pancreas cDNA library using a 2.0 kb fragment of the bovine 115kD subunit cDNA resulted in the isolation of 13 positive clones. Rescue of these clones into pBluescript allowed for T3 and T7- primed sequencing of the termini of each clone. The high degree (85-95%) of sequence identity relative to the known bovine sequence allowed for positional mapping of the clones relative to the bovine sequence. The positions of 4 of these clones used for sequencing is shown in Figure 1. Sequencing of these clones and subcloned fragments combined with designed-primer based sequencing resulted in the determination of the nucleotide sequence (BankIt #72381). Both the bovine and rat 115kD cDNAs were previously cloned and sequenced and were shown to be highly conserved, with greater than 90% primary sequence identity. We were not surprised, therefore, to see high identity to the human sequence- 97% and 95% identity to bovine and rat primary structures, respectively. Recently, a human, osteoclast-specific 115kD cDNA sequence was published which shows less than 50% primary structure identity with the bovine, rat, and our human sequences (Li *et al.* 1996). The predicted amino acid sequence of our cDNA is compared to the bovine sequence in Figure 2. The human and bovine sequences are highly conserved, particularly in the N-termini, with 100% identity over 400 amino acids, suggesting an important functional role for this largely hydrophilic domain. The osteoclast-specific sequence shows less than 50% identity to the human or bovine sequences. Although clearly the products of different genes, the human and osteoclast-specific sequences predicted open reading frames of similar size, and the hydropathy plots of the 2 proteins are nearly identical. Since V-ATPase-mediated acidification is implicated in bone reabsorption by osteoclasts, the possibility is raised that alternate 115kD subunits may be involved in tissue-specific targeting or modulation of pump activity. Consistent with this possibility, it has been shown in yeast that at least two genes code for the 115kD subunit homolog (Manolson et al. 1994).

It was previously shown by Peng et al, that the 115kD subunit is present in tissue-specific isoforms as a result of alternative splicing. An 18bp insert was shown to be present in bovine brain but was lacking in other tissues examined. The 18bp sequence codes for a protease-sensitive PEST sequence, the implication being that this might be involved in increased, protease-mediated turnover of the peptide. Since V-ATPase-induced acidification has an important role in neurotransmitter uptake and storage in synaptic vesicles, it is possible that brain-specific isoforms of the 115kD subunit exist through an alternative splicing mechanism. We were therefore interested to investigate whether similar such isoforms occur in human brain or other tissues. PCR primers flanking the region of alternative splicing seen in the bovine were generated and 28 human cDNA libraries were PCR screened. Alternative PCR products were present in human brain libraries but were absent in other human tissues examined (Figure 3). Table 1 lists the tissue libraries screened and indicates the presence or absence of alternative product. Six of nine brain libraries (including retina) showed the additional, slightly larger product, while all non-neural libraries contained only the single product of the predicted size. These data indicate a similar pattern of isoform distribution is present in human tissues as has

been described in bovine tissues. Notably, three neural libraries (medulla, putamen, and caudate) lacked the alternative product. Whether this acurately reflects isoform distribution within the brain or is a result of loss of this product sequence during cDNA preparation remains to be investigated. The alternative PCR products are currently being sequenced.

Discussion

V-ATPases

Vacuolar-type H^+ ATPases (V-ATPase) are heteromultimeric proteins found in all eukaryotic cells, and have been highly conserved among divergent species. V-ATPases couple hydrolysis of ATP to the translocation of protons across a membrane and are largely responsible for the acidification of intracellular vesicles and membrane-bound compartments. In addition, some cell types express V-ATPases at the plasma membrane, where they may play a role in pH regulation or the acidification of extracellular compartments.

The pump complex is comprised of "V_1" and "V_0" regions (by analogy with the related F_1F_0 H^+ ATPases). V_0 is a hydrophobic, integral membrane region comprised of a hexamer of 16kD subunits and single copies of 115, 45, and 39kD peptides. The 16kD hexamer is thought to form the proton "pore'. V_1 contains a hydrophillic "head", composed of three copies each of A (70kD) and B (60kD) subunits, and an intervening "stalk" with single copies of C (41kD), D (34kD), and E (33kD) subunits. Both the A and B subunits have ATP binding sites, with catalysis thought to occur on the A subunit, and the ATP binding properties of the B subunit proposed to be regulatory. The specific function of other subunits is unknown. Although ATP catalysis is dependant upon the presence of both C and E subunits, these and other subunits are thought to mediate V_1-V_0 association and/or play other regulatory roles.

The existence of different isoforms of various pump subunits has been demonstrated, and it has been proposed that these alternative isoforms may result in cell- or organelle-specific targeting or specificity of activity of V-ATPases in different tissue types or subcellular compartments. For example, osteoclasts, which constituitively express VATPase at the plasma membrane (for acidification of extracellular bone resorptive compartments), have been shown to express different isoforms of A and B (Chatterjee et al 1992) and 115 kD (Li et al 1996) subunits. These isoforms may provide for specific targeting and regulation of plasma membrane V-ATPase activity distinct from that of the "housekeeping" activity of V-ATPase in endocytic compartments, Golgi, lysozomes, etc. Alternative splicing has been shown to give rise to different isoforms of the A (Hernando et al. 1995) and 115kD (Peng et al 1994) subunits in a tissue-specific manner, and distinct genes encode different isoforms of the B (Bartkeiwitz et al. 1995) and 115kD (Manolson et al. 1994) subunits. Thus far, cell types expressing unique subunit isoforms are those in which altered VATPase activity has been demonstrated or hypothesized, i.e. extracellular compartment acidification by osteoclasts or renal epithelia, synaptic vesicle acidification in brain. In contrast, the classical, ubiquitous isoforms are present in most cell types. Demonstration that distinct genes code for alternative forms of the 115kD homolog in yeast (Manolson et al 1994) suggests that this subunit may play a role in targeting or regulation of the pump within a single cell. Other subunits with unknown function- C, D, E, 39kD, 45kD, etc., may also be involved in regulation, but well-developed models of V-ATPase regulation are currently lacking.

Plasma membrane expression

A number of cell types express V-ATPases at the plasma membrane. In these cells, including osteoclasts, macrophages, renal epithelia and neural cells, the plasmalemmal V-ATPase expression is important to some specialized cell function. Osteoclasts use the pump to acidify an extracellular bone resorptive compartment during skeletal development (Chaterjee et al 1992). Macrophages, which may utilize up to 50% of plasma membrane during phagosomal formation, constituitively express V-ATPase at the plasma membrane (David and Baron 1994), likely in order to facillitate rapid phagosomal acidification. Apical plasma membrane of renal intercalating cells is highly enriched in V-

ATPase (Blair et al 1989) for H^+ export into the urine and, in neurons, rapid acidification of plasma membrane-derived synaptic vesicles is neccesary for effective concentration of catecholamine neurotransmitters.

In addition to the aforementioned cell types, a large number of cancerous cell lines exhibit plasma membrane V-ATPase activity, as demonstrated by a bafilomycin-sensitive ability to recover from acid loads in absence of Na^+ and HCO_3^- (Martinez-Zaguillan et al. 1992). The significance of this activity in cancerous cells is not clear, but a number of possibilities exist. V-ATPases are not usually thought to be significantly involved in cytosolic pH maintenence, sinceV-ATPase activity is low with respect to the Na^+-H^+ exchanger (NHE). However, V-ATPases clearly have the potential to significantly affect cytosolic pH, especially at alkaline values, where NHE activity is diminished (Ravesloot et al. 1995). Proliferative and cancerous cells generally maintain a higher cytosolic pH than do normal cells, even within tumors where localized extracellular pH may be very acidic. Plasmalemmal V-ATPase activity could be significant in allowing cells to maintain this exagerrated pH gradient, sustaining cytosolic conditions conducive to growth, and even selecting for these cells in acidic environs.

Our attempts to visualize V-ATPase at the plasma membrane in cells expressing this activity via immunocytochemistry have been inconsistent. No cell surface staining has been detected using antibodies against the "A" or "B" subunits, while OSW-2 has been shown to stain the plasma membrane. These data suggest that there may be rapid cycling of V-ATPase-containing vesicles with the plasma membrane and that, while in the plasma membrane, the V_0 and V_1 subunits are dissociated. Such a mechanism would allow for exocytotic removal of protons from the cell, while not requiring extensive, static residence of the V-ATPase in the plasma membrane itself.

Endocytosis

All eukaryotic cells exhibit of endocytosis- the pinching off and internalizing of plasma membrane-derived vesicles along with enclosed extracellular fluid. Phagocytosis, an actin dependant formation of pseudopodia to engulf and internalize particulates is exhibited by some specialized cell types (macrophages, neutrophils, slime molds, etc), but is not common to most cells and will not be discussed here. Common to almost all cells, and of importance here, are clathrin-dependant and -independant processes of endocytosis.

Clathrin-dependant endocytosis is characterized by the formation of invaginations in the plasma membrane coated with, and probably at least in part formed by, a cagelike lattice of the structural protein clathrin (Robinson 1994). A number of cell surface receptor types, upon binding of ligand, become concentrated in these coated pits and subsequent pinching off of the pit results in the formation of clathrin coated vesicles. The observation that receptor-ligand complexes are highly concentrated in these vesicles has resulted in this process being referred to as receptor-mediated endocytosis (Goldstein et al. 1985). The clathrin coat of these vesicles rapidly disassembles, allowing for the fusion of said vesicles with early endosomes. Early endosomes are tubulovesicular membrane bound compartments of mildly acidic pH (6.0-6.8) which facillitate the uncoupling of most receptor-ligand complexes. Early endosomes are a point of significant membrane protein sorting, with many receptors and internalized plasma membrane proteins being concentrated in tubular regions of the endosome, from which vesicles pinch off and recycle to the plasma membrane, returning vacant receptors for reuse (Mellman 1996). Some receptor-ligand complexes, along with the bulk of fluid phase material and dissociated ligands, are transported to late endosomes and lysozomes, compartments of more extreme V-ATPase-mediated acidity, where degradative enzymes digest ligands and remaining vesicular contents. It is worth noting that the different "stations" in the

endocytic pathway- early and late endosomes, lysozomes ,and the vesicular or tubular extensions which connect them- are not clearly defined structural elements, since the extensive exchange of membrane and membrane-bound compartments blurs these boundaries. They are distinguished by various biochemical markers and functional attributes, such as pH. The underlying mechanisms by which certain membrane proteins are retained at one site, or are recycled back to a specific point in the pathway are not well defined, but the fact that these general components of the endocytic pathway (and the intersecting biosynthetic pathway) are discernable given the extensive bulk movement of membrane through the pathway is indicative of extensive sorting, retrieving, and recycling activity.

Much less well characterized is clathrin-independant endocytosis. Another coat protein, caveolin, is thought to mediate the formation of plasma membrane invaginations, termed caveolae, and the resulting plasma membrane-derived vesicles. While some receptor-ligand complexes (LDL receptor) are preferentially concentrated and internalized via caveolae, it is worth noting that disruption of clathrin dependant endocytosis appears to increase the rate of caveolae-mediated or other clathrin independant endocytic activity such that bulk fluid phase uptake, predominantly a result of clathrin-dependant endocytosis under normal conditions, is returned to near normal after a brief time.

Potential role in drug resistance

The activity of the V-ATPase, acidifying intracellular membrane-bound compartments as well as extracellular spaces in some cases, may have the potential to confer some resistance to a wide variety of weakly basic chemotherapeutic compounds. Simply as a function of its normal activity (acidifying endosomal compartments) the V-ATPase generates locales where weak base compounds might be sequestered due to being protonated in such low pH environs (Tannock and Rotin 1989). An increase in V-ATPase activity or increased rate of cycling between these compartments and the plasma membrane would increase a cells chance of avoiding the cytotoxic effects of such compounds (Raghunand, in preparation). In addition, the ability of some cells to survive and maintain elevated cytosolic pH in acidic environs within a tumor or elsewhere may enable some cells to avoid weakly basic therapeutics altogether, as the acidic external environment itself would sequester the protonated compounds outside the cell. Finally, the H^+ gradient generated between cytosolic and endocytic compartments by V-ATPase activity may provide energy for transport (antiport) of xenobiotics into vesicles, similar to the action of p-glycoprotein in using ATP hydrolysis to transport a wide variety of substrates.

Conclusion

Having determined the nucleotide and predicted amino acid sequences, we generated a hydropathy plot which predicts six transmembrane domains. We have now synthesized PCR primers flanking those nucleotide sequences coding for amino acid stretches predicted to be non-transmembrane-spanning domains. These sequences are currently being PCR amplified and will be ligated into the pGEX expression vector for generation of GST-fusion proteins. The expressed proteins will be used to generate antibodies against the various hydrophillic stretches of the 115kD subunit. We will also use the fusion proteins to test the specificity of OSW-2, a monoclonal antibody directed against an extracellular epitope of the 115kD subunit (Sato and Toyama, 1994). The orientation of this subunit in the membrane is not known, and we will attempt to determine this with the use of our antibodies and fusion proteins in competitive binding/uptake experiments. Our lack of success in detecting VATPase at the plasma membrane via immunocytochemistry and biotinylation of fixed or iced cells which functionally express plasma membrane VATPase activity suggests that this activity may be dynamic, i.e. a function of VATPase-containing vesicles rapidly recycling with the plasma membrane, rather than static VATPase presence in the plasma membrane. Antibodies targeting extracellular domains of the 115kD subunit will be useful reagents in uptake experiments designed to test this model. Generation of the fusion proteins neccessary to raise such antibodies is the focus of work currently in progress.

Figure 1. Positional Map of Selected Clones vs. Bovine Sequence

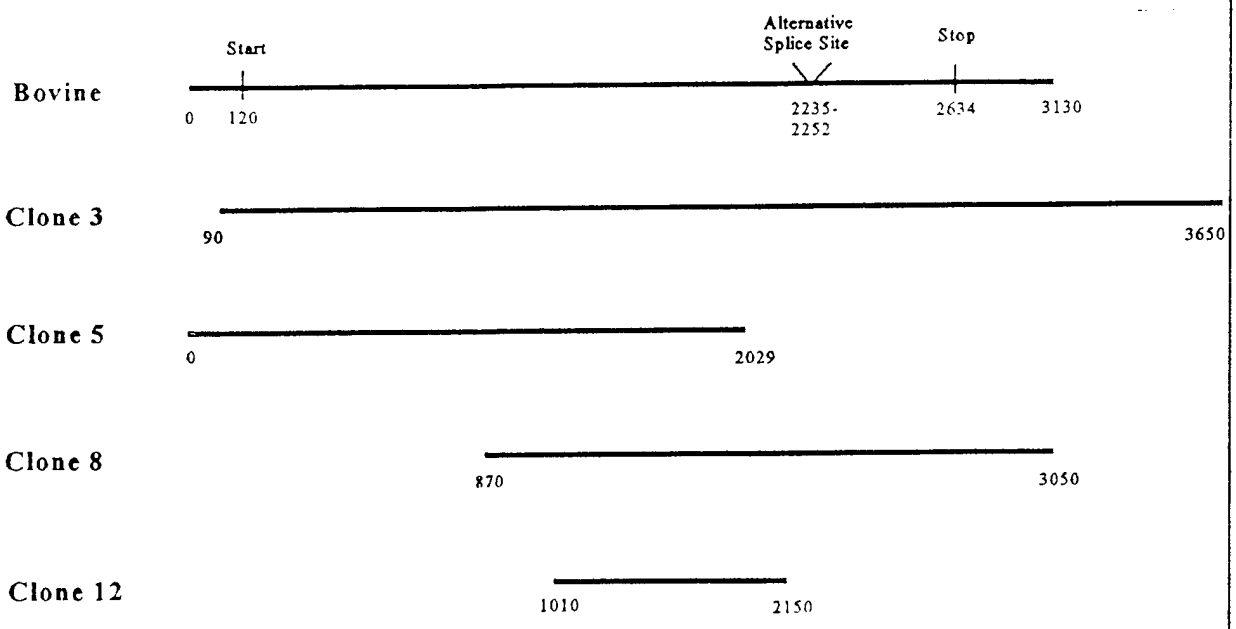

FIGURE 2. Comparison of Bovine and Human Amino Acid Sequence of 115kD Subunit (differences shaded)

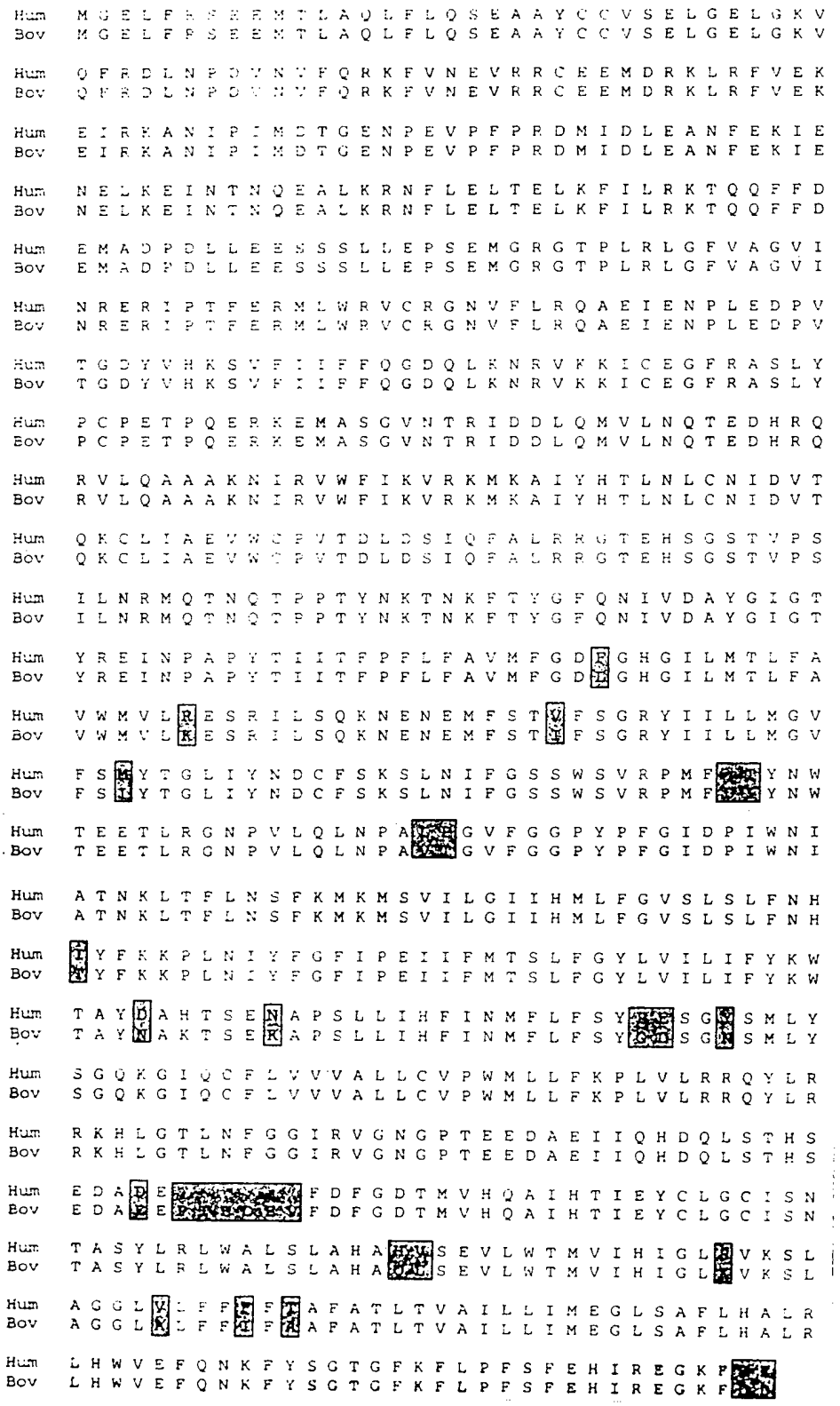

Figure 3. PCR- Alternative Splicing

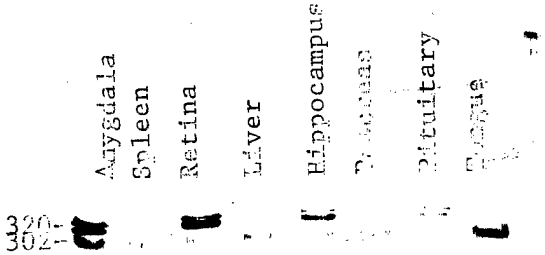

Table 1. PCR Screening of Human cDNA Libraries for Alternative Products

Tissue	Alternative Products
Globus pallidus	+
Hippocampus	+
Cerebellum	+
Amygdala	+
Pituitary	+
Caudate	–
Putamen	–
Medulla	–
Retina	+
Breast carcinoma	–
Epidermis	–
Fat	–
Hepatoma	–
Liver	–
Placenta	–
Foreskin	–
Bone marrow	–
Lung	–
Kidney	–
Adrenal	–
Melanoma pool	–
Glioblastoma pool	–
Leukemia	–
Endothelium	–
Skeletal muscle	–
Colon carcinoma	–
Breast carcinoma pool	–

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Li,YP, Chen,W, Stashenko,P. (1996) *Biochem. and Biophys. Res.*218, 813-821

Manolson,MF, Wu,B, Proteau,D, Yailloon,B, Roberts,BT, Hoyt,MA, Jones,EW.(1994),. *J. Biol. Chem.*269 14064-14074.

Peng,SB, Crider,BP, Xie,XS, Stone,DK (1994) *J.Biol.Chem.*269, 17262-17266.

Sato,SB and Toyama,S (1994) *J. Cell Bio.*127, 39-52.

Appendix A Revised Statement of Work

Year1. **Clone and sequence the human 115kD subunit**
- **1.1 Library screen using bovine 115kD subunit cDNA probe**
- **1.2 Sequence clones**
- **1.3 PCR-screen of libraries for tissue-specific isoforms**
- 1.4 Identify PCR products

Year2. Characterization of human 115kD subunit
- 2.1 Generate fusion proteins using putative extracellular domains
  - 2.1.1 Generate antibodies targeting these extracellular domains
  - 2.1.2 Use fusion proteins to test specificity of OSW-2, a monoclonal antibody vs. a 115kD extracellular epitope
- 2.2 Epitope map the 115kD subunit using fusion protein generated antibodies in whole cells
- 2.3 Determine the chromosomal location of gene coding for the 115kD subunit by Southern and FISH
- 2.4 Determine response of 115kD expression to acidic environment in different cell types

Year3. Antibody uptake experiments
- **3.1 Work out conditions of uptake time course experiments**
- 3.2 Determine uptake time constants for drug-resistant and drug-sensitive cells
- 3.3 Determine effects of pharmacologically blocking endosomal turnover on:
  - 3.3.1 Uptake of antibody against 115kD subunit in drug-resistant and -sensitive cells
  - 3.3.2 Plasma membrane V-ATPase activity
  - 3.3.3 Drug resistance

Bold type indicates work accomplished

Appendix B Response to Specific Reviewer Concerns

!. Contractual issues

Reviewer statement: "...report should contain a justification for the large deviation from the proposed experiments..." and "..a revised SOW (should) be submitted..."

As a graduate student, I hope to obtain a Ph.D. My graduate committee found my initially proposed work to be sorely lacking. The immunocytochemistry experiments were deemed to be too descriptive, and biotinylation a poor means of answering relevant questions. In short, I've generated a much stronger aproach and set of experiments, as should be clear from the revised SOW enclosed (Appendix A).

Reviewer statement: "...data from the PCR experiments should have been included..."

I've now included an example of this data in Figure 3.

2. Technical issues

Reviewer statement: "The rational for examining expression in cDNA libraries as opposed to tissue samples is unclear."

cDNA libraries for the 28 tissues examined were readily available. This is the only rational. This particular focus (tissue-specific alternative splicing) is not central to my work and will not be pursued.

Reviewer statement: "... is the common isoform ubiquitously expressed in tissues?"

Yes, as seen in the newly included Figure 3.